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14. ABSTRACT

We seek to understand four basic questions related to epileptogenesis: 1) What is the role of mTOR dysregulation in epileptogenesis in the developing brain? 2) What are the molecular mechanisms downstream of mTOR hyperactivation that trigger epileptogenesis in developing brains? 3) What are the long-term pharmacological treatments to prevent the development or progression of seizures? 4) What are biomarkers for epileptogenesis and the prognostic biomarkers for disease progression? We have established a neuroglial tuberous sclerosis complex 1(TSC1)-deficient mouse model in which spontaneous seizures begin at 2.5 months of the age and progress into 7-8months. This time course allows the analysis of molecular changes before and after the onset of spontaneous electrographic and behavioral seizures and provide reliable biomarkers for epileptogenesis and the progression of epilepsy. We have also developed a neuronal-specific mouse model, deleting Tsc1 by driving cre from the CAMK2 promoter. We propose gene expression profiling, electrophysiological, biochemical, immunohistochemical and behavioral studies.

15. SUBJECT TERMS

Nothing listed

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INTRODUCTION

Tuberous Sclerosis Complex (TSC) is a multisystem genetic disorder caused by germline mutations in the TSC1 or TSC2 genes, which form a regulatory complex responsible for limiting the activity of an important intracellular regulator of cell growth and metabolism known as mammalian target of rapamycin complex 1 (mTORC1). The pathological hallmark of TSC brains are cortical tubers, characterized by disorganized cortical lamination, aberrant dendritic arbors and axonal projections, astrocyte gliosis and abnormal cell morphology (ie, dysplastic and heterotopic neurons and giant cells). mTOR hyperactivity contributes to developmental brain malformations associated with seizures and cognitive and psychiatric deficits. In our studies, we want to understand the molecular changes that occur in neurons and astrocytes as a result of a deletion of TSC1, using a mouse model. These findings will help us understand molecular mechanisms underlying cellular changes in TSC and underlying epilepsy. Hopefully, a knowledge of these mechanisms will aid in a rational development of therapies.

KEYWORDS

Tuberous Sclerosis, Epilepsy, Neurons, Astrocytes, EEG, mTOR, mRNA translation,

ACCOMPLISHMENTS

The major goals of the project:

Specific Aim 1: To Determine the effects of TSC1 deletion in the Tsc1$^{CamkII\text{-}Cre}$CKO and Tsc1$^{mGFAP\text{-}Cre}$CKO mice mice on epileptogenesis and progress of seizures. Inactivation of the Tsc1 or Tsc2 gene in different subtypes of brain cells, including neurons, glia or progenitor cells, all cause spontaneous seizures in animal models, suggesting that both neuronal and astroglial cells are active elements in the epileptic brain. In this aim, we hypothesize that TSC1 deletion in astrocytes and a subset of late-onset forebrain pyramidal neurons underlie the changes in preclinical epileptic EEG activities which progress into clinical behavioral seizures. To test this hypothesis, we will monitor spontaneous electrographic and behavioral seizures by continuous video/EEG recording in free-moving mice. We will perform Video-EEG tracking of epilepsy and epileptogenesis in both neuronal specific *Tsc1*$^{CamkII\text{-}Cre}$CKO and neuroglia *Tsc1$^{mGFAP\text{-}Cre}$CKO* mouse models.

Specific Aim 2. To analyze the molecular changes downstream of TSC deficiency in astrocytes and neurons that are related to the initiation and progression of seizures. The initiation of the first seizure and the progression of the disease after the onset of seizures are two distinct processes. The identification of molecular changes for these two processes may provide biomarkers for epilepsy prevention and disease modification. We hypothesize that mTOR controls the translation of a specific repertoire of neuronal and astrocytic genes, which cooperatively regulate the initiation and progression of epilepsy. Translational ribosomal profiling will be used to investigate gene expression in the both *Tsc1$^{CamkII\text{-}Cre}$CKO* and *Tsc1$^{mGFAP\text{-}Cre}$CKO* mice, before and after the occurrence of spontaneous seizures. The gene expression signatures in these mice will be compared with previous report of transcriptional changes in response to chemical induced epilepsies and find common developmental changes in pathways responsible for epileptogenesis and for the progression of epilepsy.

Specific Aim 3. Determine the effects of mTOR inhibitor on the initiation and progression of seizures and on the molecular changes that accompany the initiation and progression of seizures. Preclinical studies demonstrate that mTOR inhibitors can effectively treat seizures in mice with established epilepsy related to mTOR pathway hyperactivation. However, the downstream mechanisms responsible for these effects have not been proven. In this aim, we will test the efficacy of potential treatments with mTORC1 inhibitors and identify molecule players that respond to or do not respond to mTORC1 inhibitors. We will examine the effects of rapamycin on the molecular changes associated with the EEG/seizure activity, focusing on those genes and pathways we have found to be significantly altered in studies of Specific Aim 2.

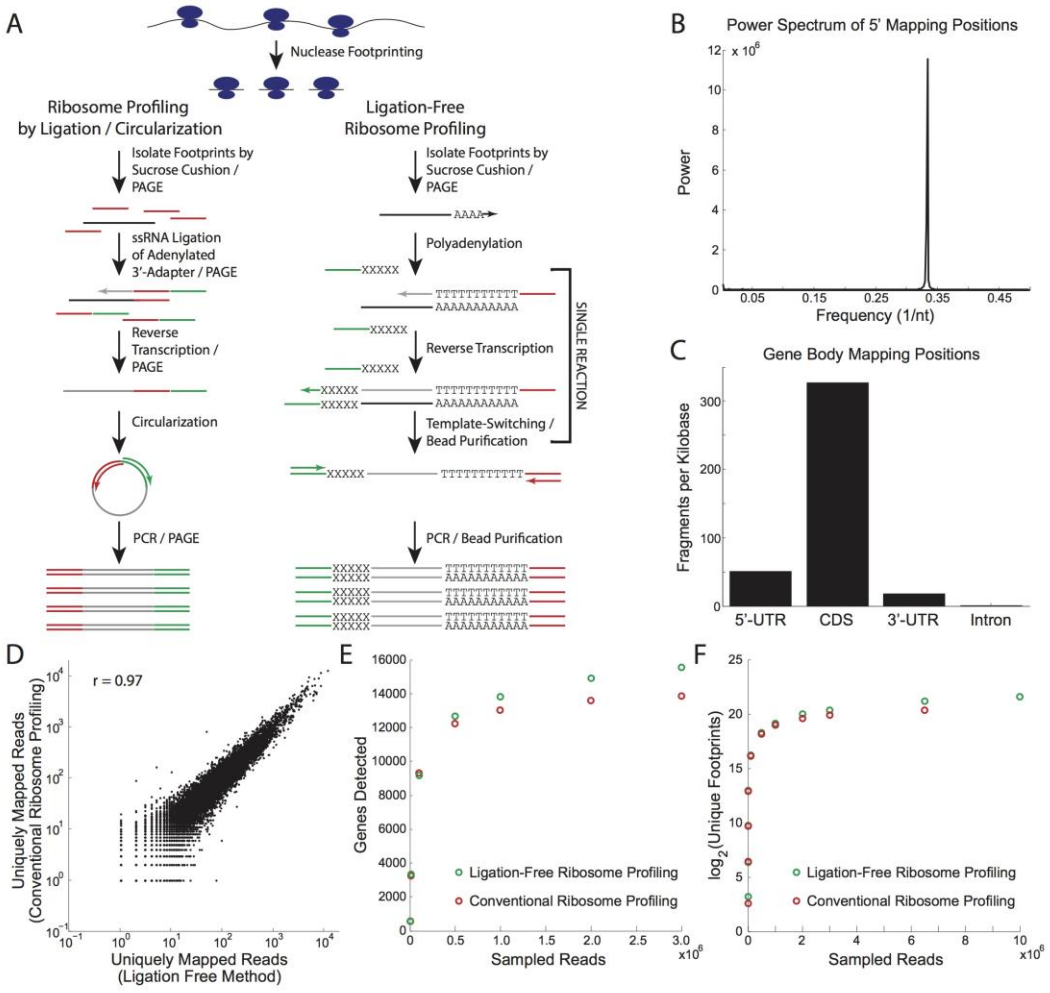
What we have accomplished:

1. We have developed, verified, and reproduced the critical molecular assays required for this work.

New Technology for Highly-Sensitive Ribosome Profiling in Tissue

In addition to conducting initial ribosome profiling and RNA-Seq experiments in our TSC mouse model, we have also worked to significantly improve the ribosome profiling technique itself during the first funding period. Specifically, we have developed a new ribosome profiling technique called ligation-free ribosome profiling that exhibits reduced bias and ~100-fold higher sensitivity than the originally reported method (**Fig. 1**). We recently reported this new technique and its application to cell type-specific translational profiling in the brain in *Genome Biology* (Hornstein et al, 2016). Ligation-free ribosome profiling is particularly useful for tissue-based studies and cell type-specific profiling experiments where material is limiting, and we have already deployed this new method for the ongoing studies described here.

Fig. 1. Comparison of Ligation-Free Ribosome Profiling to Conventional Methods. A) Schematic of the steps involved in conventional ribosome profiling and ligation-free ribosome profiling. B) The power spectrum of 5' mapping positions from CDS reads resulting from ligation-free ribosome profiling method shows clear three-base periodicity that is characteristic of ribosome profiling libraries and reflects the single-codon translocation of the ribosome. C) Gene body distribution of mapped reads from ligation-free ribosome profiling show strong preference for coding-sequence, an additional property inherent to ribosome profiling libraries. D) Comparison of the number of uniquely mapped reads per gene in libraries generated with footprints from mouse-forebrains prepared with conventional ribosome profiling strategy and the ligation-free method; the Pearson correlation r = 0.97 indicates a concordance between the two methods. E) Saturation analysis showing the number of unique genes detected following downsampling of ligation-free ribosome profiling and conventional ribosome profiling. F) Saturation analysis showing the number of unique footprints detected following downsampling of ligation-free ribosome profiling and conventional ribosome profiling.



Ligation-Free Ribosome Profiling in CamK2a-Cre Tsc1$^{fl/fl}$ Mice following Rapamycin Treatment

One of the major goals of the proposed studies is to assess the alterations in protein synthesis regulation that occur in TSC in response to rapamycin treatment. We reasoned that the phenotypic effects of mutations in Tsc1 and the response to rapamycin treatment would be most significant in neurons. Therefore, we crossed Tsc1$^{fl/fl}$ mice with CamK2a-Cre mice to generate offspring with Tsc1 deleted in CamK2a-expressing, excitatory neurons. We then conducted ribosome profiling and RNA-Seq on brain tissue isolated from P30 animals both with and without rapamycin treatment.

In analyzing the results, we first sought to validate our approach and the efficacy of our rapamycin treatment. It is well-established that specific inhibition of mTORC1 results in sharp translational downregulation of a set of ribosomal proteins and translation factors through the 4EBP-EIF4E signaling axis. This interaction is facilitated by 5'-terminal oligopyrimidine (5'-TOP) tracts, which are CT-rich motifs common to the 5'-UTRs of target mRNAs. We recently reported that translational control of these genes in the murine brain using ribosome profiling and a potent, competitive mTOR inhibitor (Hornstein et al, 2016). However, rapamycin is significantly less potent and allosteric. We computed genome-wide translation efficiencies for the treated and untreated animals and ranked all genes based on their translation efficiency fold-change. Using gene set enrichment analysis (GSEA), we found that genes containing the TOP-motif are significantly enriched (p_{adj} < 0.00001) among the genes that are translationally downregulated by rapamycin treatment (**Fig. 2A**). Hence, we concluded that the rapamycin treatment and our measurements were successful.

We next sought to determine whether there are cell type-specific alterations in translation efficiency in response to rapamycin treatment. Our initial hypothesis is that these would be largely restricted to neurons, since the Tsc1 deletion occurs only in Camk2A-expressing cells. **Figs. 2B-C** show GSEA for translation efficiency fold-change for neuron-specific and oligodendrocyte-specific genes, respectively. Surprisingly, while there are some neuron-specific genes that are translationally downregulated by rapamycin, the effects are much more global and dramatic among genes specific to myelinating oligodendrocytes (p_{adj} < 0.00001). These results are quite exciting and interesting, but further studies will be required to a) confirm this outcome by the addition of more replicates and b) determine whether there is an actual non-cell autonomous effect on oligodendrocyte phenotype.

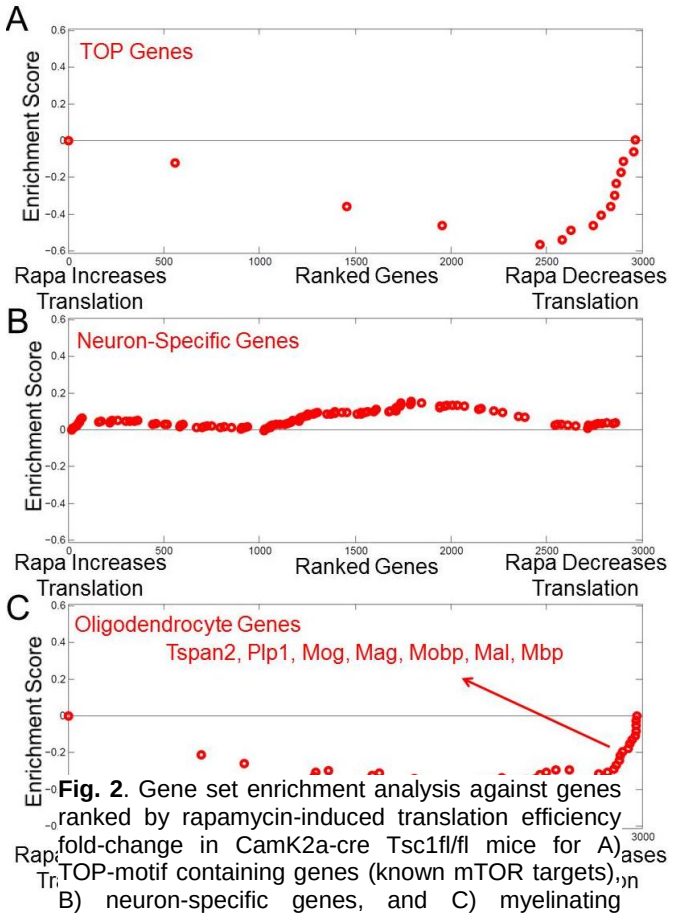


Fig. 2. Gene set enrichment analysis against genes ranked by rapamycin-induced translation efficiency fold-change in CamK2a-cre Tsc1fl/fl mice for A) TOP-motif containing genes (known mTOR targets), B) neuron-specific genes, and C) myelinating oligodendrocyte-specific genes.

2. We have bred many of the mice required for the molecular studies and frozen brains for molecular analysis. These mice have had the TSC1 gene deleted from excitatory neurons in the cortex. They include appropriate controls as well as mice treated with rapamycin, an mTOR inhibitor that allows the mice to survive without seizures.

Sample ID	Genotype	Treatment	Mouse ID	Age at collection
TGS-001	TSC1CKO;Rpl22flox/flox (Knockout)	Untreated Control	GT78.1_R1	P30
TGS-002			GT78.1_R2	P30
TGS-003			GT78.1_NA	P30
TGS-004			GT76_R3	P30
TGS-005		Rapamycin treated	GT75_R1	P30
TGS-006			GT77.2_R2	P30
TGS-007			GT78.1_R3	P30
TGS-008			GT78_R3-	P30
TGS-009	Rpl22flox/flox:CamKCre+ (Control)	Untreated Control	#19_R1	P30
TGS-010			#20_R4	P30
TGS-011			#22_R5	P30
TGS-012			GT77_L4	P30
TGS-013		Rapamycin treated	GT75_R2	P30
TGS-014			GT75_L2	P30
TGS-015			GT77.1_R5	P30
TGS-016			GT77_R5	P30

During the next reporting period we will begin to analyze the molecular changes in these brains, as described in the grant application.

IMPACT
Nothing to report

CHANGES/PROBLEMS
Nothing to report

PRODUCTS
Nothing to report

PARTICIPANTS
Individuals who have worked on the project: No change.
Change in other support: Nothing to report.
Other organizations: Nothing to report